Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9543–9547, November 1991
Biochemistry

Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: Comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

(anticarcinogen/*Brassica oleracea*/indolo[3,2-*b*]carbazole)

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Communicated by Bruce N. Ames, July 25, 1991

ABSTRACT **Indole-3-carbinol (I3C) is a secondary plant metabolite produced in vegetables of the *Brassica* genus, including cabbage, cauliflower, and brussels sprouts. I3C is both an anti-initiator and a promoter of carcinogenesis. Consumption of I3C by humans and rodents can lead to marked increases in activities of cytochrome P-450-dependent monooxygenases and in a variety of phase II drug-metabolizing enzymes. We have reported previously that the enzyme-inducing activity of I3C is mediated through a mechanism requiring exposure of the compound to the low-pH environment of the stomach. We report here the aromatic hydrocarbon responsiveness-receptor K_d values (22 nM–90 nM), determined with C57BL/6J mouse liver cytosol and the *in vitro*- and *in vivo*-molar yields (0.1–6%) of the major acid condensation products of I3C. We also show that indolo[3,2-*b*]carbazole (ICZ) is produced from I3C in yields on the order of 0.01% *in vitro* and, after oral intubation, *in vivo*. ICZ has a K_d of 190 pM for aromatic hydrocarbon responsiveness-receptor binding and an EC_{50} of 269 nM for induction of cytochrome P4501A1, as measured by ethoxyresorufin O-deethylase activity in murine hepatoma Hepa 1c1c7 cells. The binding affinity of ICZ is only a factor of 3.7×10^{-2} lower than that of the highly toxic environmental contaminant and cancer promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. ICZ and related condensation products appear responsible for the enzyme-inducing effects of dietary I3C.**

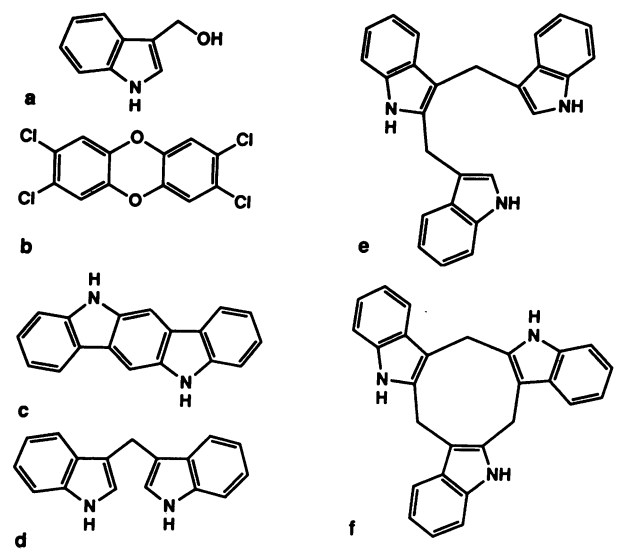

FIG. 1. (Compound **a**) I3C. (Analog **b**) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). (Analog **c**) Indolo[3,2-*b*]carbazole (ICZ). (Analog **d**) 3,3′-Diindolylmethane. (Analog **e**) 2-(Indol-3-ylmethyl)-3,3′-diindolylmethane (LT). (Analog **f**) 5,6,11,12,17,18-Hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole (CT).

Indole-3-carbinol (I3C) (Fig. 1 compound **a**) is an autolysis product of glucobrassicin (3-indolylmethyl glucosinolate), a compound that occurs naturally in large amounts in a number of vegetables of the *Brassica* genus (e.g., cabbage, 0.1–1.9 mmol/kg of fresh weight; cauliflower, 0.1–1.6 mmol/kg; and brussels sprouts, 0.5–3.2 mmol/kg) (1–3). I3C has received considerable attention as a dietary modulator of carcinogenesis (4). When administered before carcinogen exposure, I3C reduces both the incidence of neoplasia and the formation of covalent adducts of carcinogen with DNA (5–7). When administered after carcinogen exposure, I3C increases neoplastic outcome and, thus, promotes carcinogenesis (8–10).

The mechanism(s) by which I3C modulates carcinogenesis may be related to its potency as an inducer of enzymes involved in the metabolism of carcinogens and other foreign chemicals (5). In rodent models, oral administration of low levels of I3C significantly increased activities of epoxide hydrolases, quinone reductase, and cytochrome P4501A1 (CYP1A1¶)-dependent monooxygenase, and at high levels increases in glutathione S-transferases were observed (5, 12, 13). In humans, the effects of I3C appear similar. Human volunteers exposed to purified I3C showed increases in the activity of estradiol 2-hydroxylase (14). Human volunteers consuming *Brassica* vegetables also showed marked increases in the oxidative metabolism of phenacetin and antipyrine (15), as well as in the glucuronidation of acetaminophen (16).

We have been interested in the mechanism by which I3C induces CYP1A1-dependent monooxygenase activity and in its properties as a modulator of carcinogenesis. Several experimental observations have provided support for the idea that I3C is activated via an acid-catalyzed reaction occurring in the low-pH environment of the stomach. Evidence to support this hypothesis includes the observations that (*i*) oral, but not i.p., administration of I3C led to an induction of hepatic CYP1A-dependent monooxygenase activities (12, 17); (*ii*) acid treatment of I3C generated a reaction mixture that induced monooxygenase activity after i.p. and oral administration (12); and (*iii*) results of structure–activity

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Abbreviations: I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; LT (linear trimer), 2-(indol-3-ylmethyl)-3,3′-diindolylmethane; CT (cyclic trimer), 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; Ah, aromatic hydrocarbon responsiveness.

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¶Cytochrome P-450 nomenclature is according to Nebert *et al.* (11).

studies indicated that indoles unstable at acidic pH have greater potency as inducers of monooxygenase activity than do indoles stable at acidic pH (12). In this report, we present results of experiments designed to further describe the mechanism(s) of action of I3C. First, we show that I3C is converted in potentially significant yields *in vivo* and *in vitro* to ICZ and other methyleneindole condensation products (Fig. 1). Second, we report the aromatic hydrocarbon responsiveness (Ah)-receptor-binding characteristics of these indole derivatives, and third, we characterize the potency of ICZ as an inducer of CYP1A1 activity in murine hepatoma cells, Hepa 1c1c7.

MATERIALS AND METHODS

Chemicals. We purchased indole-3-carboxaldehyde, indole-3-acetonitrile, and I3C from Aldrich. These indoles were recrystallized immediately before use. We prepared and recrystallized 3,3′-diindolylmethane (Fig. 1 analog **d**) and ICZ (Fig. 1 analog **c**) according to published methods (18, 19). We isolated 2-(indol-3-ylmethyl)-3,3′-diindolylmethane (LT) (Fig. 1 analog **e**) and 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole] (CT) (Fig. 1 analog **f**) from the acid reaction mixture of I3C by HPLC using conditions described below. Purities of trimer samples were established by HPLC analyses as >98%. Identities were established by comparing NMR and mass spectra to published data (20, 21). TCDD (Fig. 1 analog **b**) was a gift from B. N. Ames (University of California, Berkeley, CA). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and radioligand [2-^{125}I]7,8-dibromodibenzo-*p*-dioxin were gifts from A. Poland (McArdle Laboratory for Cancer Research, Madison, WI). We purchased resorufin from Aldrich. We prepared and purified ethoxyresorufin according to published procedures (22).

Analysis of Acid Condensation Products of I3C *in Vitro* and *in Vivo*. An acid reaction mixture was generated at ambient temperature by treating a stirred aqueous solution of I3C (1 mg/ml) with 1 M hydrochloric acid. The reaction was neutralized with 0.25 M aqueous ammonia, diluted with tetrahydrofuran, 60:40, and analyzed by HPLC using a C_{18} bonded-phase column with acetonitrile in water as the mobile phase. To monitor production of most condensation products, we used UV absorption at 280 nm and calibration against known standards. We used a fluorescence detector for routine analyses of ICZ. HPLC-MS (Hewlett–Packard model HP5988) was used to confirm peak identity for ICZ and 3,3′-diindolylmethane.

To monitor production of condensation products from I3C in the gastrointestinal tract, we dosed male Sprague–Dawley rats by oral intubation with I3C (73.5 mg/kg of body weight) in corn oil, euthanized them, and then excised their gastrointestinal tracts and contents. The ethyl acetate extracts of sucrose/phosphate (pH 7.4) homogenates were then filtered through a nylon membrane and analyzed by HPLC. I3C and the acid reaction mixture products are stable under these extraction conditions. Thus, products identified *in vivo* are not produced as artifacts of the analytical procedures.

Characterization of I3C Condensation Products as Agonists of the Ah Receptor. To determine affinity of compounds for the Ah receptor, we conducted competitive binding experiments with the radioligand [2-^{125}I]7,8-dibromodibenzo-*p*-dioxin. Bound and free radioligand were separated by the charcoal-adsorption assay with Ah receptor prepared from C57BL/6J mouse liver cytosol (23).

Analysis of Induction of CYP1A1-Dependent Activity. To compare the capacity of a purified compound or reaction mixture to induce CYP1A1 activity, we monitored the induction of ethoxyresorufin *O*-deethylase in cultured murine hepatoma cells (24). Results of previous experiments have shown that I3C treatment increases the levels of *CYP1A1* gene transcripts in the cytosol (25). Briefly, confluent Hepa 1c1c7 cells were treated with different concentrations of the test materials dissolved in dimethyl sulfoxide (final dimethyl sulfoxide concentration of 1.5% (vol/vol) in the growth medium). After 2 days of incubation at 37°C, 3×10^6 cells were suspended in ≈1.5 ml of isotonic NaCl/phosphate buffer at pH 7.4 in a 1-cm-square fluorometer cell. To the cell suspension we then added 0.5 ml of a solution of ethoxyresorufin in the same buffer to make the final ethoxyresorufin concentration 2.5 μM in the cuvette. To monitor resorufin production, we recorded the fluorescence produced at 586 nm from excitation at 510 nm. We calibrated the fluorescence measurements with authentic resorufin.

RESULTS AND DISCUSSION

HPLC analysis indicated that within 10 min in aqueous acid I3C is converted to a complex mixture. The three most prevalent UV-absorbing compounds in the mixture were LT, CT, and 3,3′-diindolylmethane (Fig. 1). Molar yields of these compounds were in the range of 2–6% of original I3C. Production of 3,3′-diindolylmethane and LT by acid treatment of I3C is consistent with previous findings (20). Further analysis of the acid reaction mixture of I3C using HPLC with fluorescence detector indicated ICZ (Fig. 1). This discovery is of particular interest because ICZ is nearly isosteric with TCDD and binds with high affinity to the Ah receptor (26). Thus, ICZ was probably a potent inducer of CYP1A1 (27–30). The evidence that ICZ is a component of the acid reaction mixture of I3C is as follows: (*i*) no ICZ peak was detectable by HPLC analysis of purified I3C; (*ii*) the presumed ICZ peak in the chromatogram of the acid reaction mixture had the same retention time and peak shape as did authentic ICZ under a range of HPLC elution conditions (Fig. 2); (*iii*) chromatographic analysis showed that spiking of the reaction mixture with authentic ICZ appropriately increased the area of the presumed ICZ peak, and no additional peaks were produced (data not shown); (*iv*) the fluorescence emission and excitation spectra of the presumed ICZ peak of the reaction mixture were identical to those of the authentic compound (Fig. 3); and finally, (*v*) HPLC-MS analyses for *m/z* 256 and 128, the two major ions present in the electron-

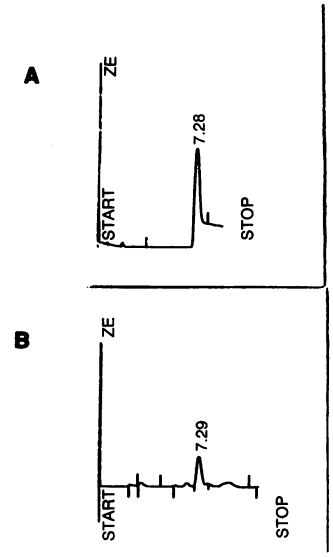

FIG. 2. (*A*) Chromatogram of ICZ (C_{18} stationary phase with 65% acetonitrile in water as eluting solvent) detected with fluorescence excitation at 335 nm and emission at 405 nm. (*B*) Chromatogram of the acid reaction mixture of I3C with fluorescence detection.

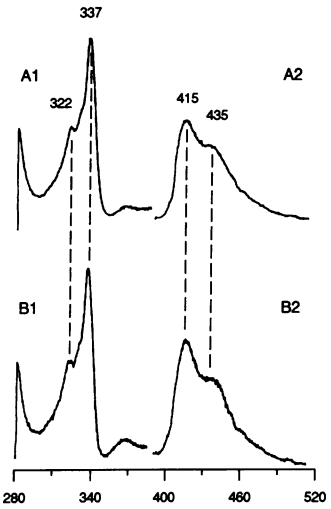

FIG. 3. Fluorescence excitation and emission spectra for the presumed ICZ peak (*A1* and *A2*, respectively) from the acid reaction mixture of I3C and from authentic ICZ (*B1* and *B2*, respectively). Spectra were obtained by scanning the HPLC peaks stopped in the detector flow cell. The fluorescence emission was measured at 415 nm in *A1* and *B1*, and the excitation was 335 nm in *A2* and *B2*.

impact mass spectrum of the authentic ICZ, indicated these masses in the proper ratio in the mass spectrum of the presumed ICZ peak (Fig. 4).

Quantitative analysis by HPLC indicated that *in vitro* yields of ICZ increased slowly with time. After 10 min of treatment with acid, the ICZ molar yield was 0.0002%; after 48 hr, the ICZ yield was 0.002%. However, when the acid-treatment mixture produced after 10 min was neutralized and diluted with tetrahydrofuran, ICZ yields were as high as 0.0075% at 20 hr and 0.0090% at 48 hr.

To determine whether the acid-condensation products are generated *in vivo*, we analyzed gastric and intestinal contents of rats after oral administration of I3C. Five hours after oral gavage with I3C, 3,3′-diindolylmethane, LT, and CT were readily detected in the small intestine (data not shown). Molar yields were ≈0.1–0.34% from I3C. ICZ was present in molar yields of 0.0016% in stomach tissue and contents of stomach and 0.0011% in contents of small intestine (Fig. 5). Twenty hours after I3C treatment, the yield of ICZ in stomach tissue and contents was only 0.0001%, and much higher yields were found in the contents of cecum (0.0088%) and in feces (0.0010%). These 20-hr figures suggest a minimum total *in vivo* yield for ICZ on the order of 0.01%. None of these values is corrected for recovery and, therefore, they reflect minimum levels of ICZ and other condensation products in the samples. The identities of the ICZ and 3,3′-diindolylmethane produced *in vivo* were confirmed by comparison of their HPLC retention times, fluorescence or UV spectra, and HPLC-MS behaviors with those of the authentic compounds.

To begin to evaluate the biological activities of I3C acid products, we compared the Ah receptor-binding affinities of the more prevalent condensation products to those of I3C and related indole monomers (Table 1, Fig. 6). In these experiments, TCDD or TCDF, two of the most potent Ah-receptor agonists known, were used as positive controls. Our results were consistent with previous findings (26, 27) but indicated that ICZ binds to the Ah receptor with K_d of 190 pM, which is similar but clearly different from the K_d for TCDD (7.1 pM). This result indicates that the receptor-binding affinity for ICZ is only 3.7×10^{-2}- and 8.4×10^{-2}-fold lower than those of TCDD and TCDF, respectively. LT, CT, and 3,3′-diindolylmethane bind with considerably less affinity and had K_d values of 22 nM, 62 nM, and 90 nM, respectively. These

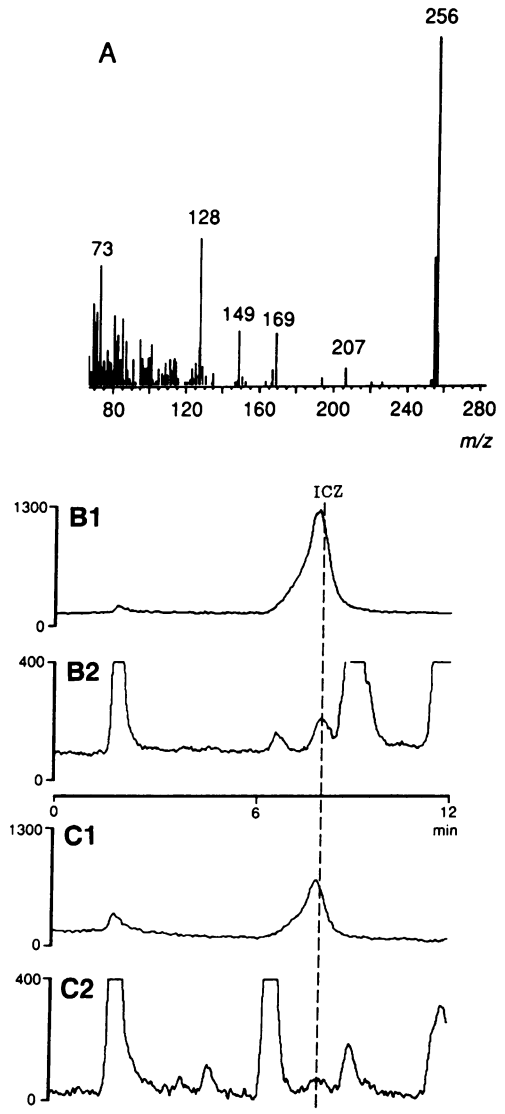

FIG. 4. (*A*) Electron impact mass spectrum of ICZ standard. (*B1*) Chromatogram of ICZ (C_{18} stationary phase with 60% acetonitrile in water as eluting solvent) detected using electron impact single-ion monitoring at *m/z* 256. (*B2*) Chromatogram of the acid reaction mixture of I3C detected with single-ion monitoring at *m/z* 256. (*C1*) Chromatogram of ICZ detected with single-ion monitoring at *m/z* 128. (*C2*) Chromatogram of the acid reaction mixture of I3C detected with single-ion monitoring at *m/z* 128. Vertical axes in *B* and *C* chromatograms represent adjusted ion counts measured for the monitored *m/z* values.

receptor-binding affinities are, thus, between 3×10^{-4}- and 8×10^{-5}-fold lower than that of TCDD.

We found that the simple 3-substituted indoles I3C, indole-3-carboxaldehyde, and indole-3-acetonitrile bound to the Ah receptor very weakly, if at all. Given the high concentrations of these indoles required to displace the radioligand, it seems probable that the observed competition may result from a trace contaminant in the indole preparation or from the generation of condensation products in solution during the 18-hr incubation time. In support of our contention that I3C is not an Ah-receptor agonist, we observed that, of all indoles tested, I3C has the lowest binding affinity for the Ah receptor. The relative binding affinities of these indoles sharply contrast with the biological potency of these congeners as inducers of monooxygenase activity in the whole animal, where I3C is the most potent inducer (12). These observations indicated that none of the simple 3-substituted indoles, including I3C, is an agonist of the Ah receptor *in vivo*.

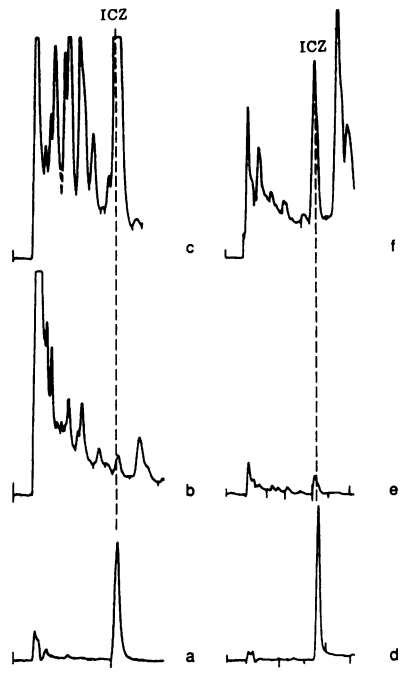

FIG. 5. HPLC (column, C_{18} stationary phase with 65% acetonitrile in 31 mmol/liter of ammonium phosphate buffer as eluting solvent; detector, fluorescence emission at 405 nm and excitation at 335 nm) chromatograms of ICZ (*a*) (detector scale at 4×); cecal contents of control rat in 20-hr group (*b*) (sample undiluted); cecal contents of I3C-treated rat in 20-hr group (*c*) (sample diluted 1:10); ICZ (*d*) (detector scale at 1×); stomach contents of control rat in 5-hr group (*e*) (sample diluted 1:10); and stomach contents of I3C-treated rat in 5-hr group (*f*) (sample diluted 1:10).

Our results from ethoxyresorufin *O*-deethylase (EROD) induction experiments in Hepa 1c1c7 cells indicated that the acid reaction mixture produced maximum induction at 100 μM (I3C equivalents), and above this dose it was highly toxic to the cells. The EC_{50} value for the reaction mixture was at least 70 μM. By contrast, the EC_{50} values for ICZ and TCDD were considerably less at 260 ± 50 nM and 36 ± 12 pM, respectively (Fig. 7). Neither ICZ nor TCDD showed signs of toxicity even at the higher doses where monooxygenase activity declined. Our value for the EC_{50} for TCDD in Hepa 1c1c7 cells is similar to the value reported by Israel and Whitlock (33).

The characteristics of these substances, as agonists of the Ah receptor, may be summarized as follows: simple 3-substituted indoles, such as I3C, do not bind to the Ah receptor with high affinity and may not be significant agonists of the Ah receptor *in vivo*. Upon contact with acid, both *in vitro* and *in vivo*, a series of indole condensation products is generated

Table 1. Binding affinities for the Ah receptor

Compound	K_d, M	Relative binding affinity
TCDD	7.1×10^{-12}	1.00
TCDF	1.6×10^{-11}	0.44
ICZ	1.9×10^{-10}	3.7×10^{-2}
LT	2.2×10^{-8}	3.3×10^{-4}
CT	6.2×10^{-8}	1.1×10^{-4}
3,3'-Diindolylmethane	9.0×10^{-8}	7.8×10^{-5}
Indole-3-carboxaldehyde	5.1×10^{-6}	1.4×10^{-6}
Indole-3-acetonitrile	7.6×10^{-6}	9.3×10^{-7}
I3C	2.7×10^{-5}	2.6×10^{-7}

The competitive binding assay was done as described (31). The true K_d of the radioligand was assumed to be 6.5×10^{-12} (31); the K_d for each compound was determined by the method of Linden (32).

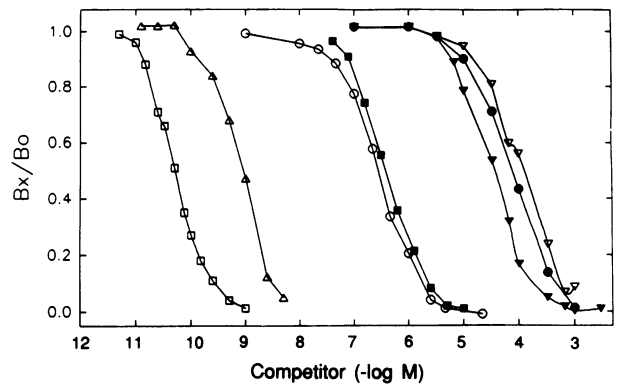

FIG. 6. Competition of TCDD and I3C condensation products for the specific binding of [2-^{125}I] 7,8-dibromodibenzo-*p*-dioxin to the Ah receptor. The competition-binding assay was done as described. Assay conditions were as follows: total radioligand concentration of 5–10 pM, total receptor concentration of 20 pM, and various concentrations of competitor (abscissa). Specific binding was determined on 1-ml reaction volumes after an 18-hr incubation at 4°C. Ordinate is B_x/B_o, specifically bound radioligand in the presence of a given amount of competitor (B_x) divided by specifically bound radioligand in the absence of competitor (B_o). Compounds were dissolved in dimethyl sulfoxide and added to the incubation in 5-μl volumes. Each data point represents the average value from at least two determinations. □, TCDD; △, ICZ; ○, CT; ■, 3,3'-diindolylmethane; ▼, indole-3-carboxaldehyde; ●, indole-3-acetonitrile; ▽, I3C. Curves for TCDF and LT are similar in shape and closely overlap curves for TCDD and CT, respectively, and were omitted for clarity.

from I3C. This reaction mixture is composed primarily of indole condensation products, which apparently bind weakly to the Ah receptor. However, whether this weak binding activity is a property of the major oligomers themselves or is due to small amounts of ICZ produced during the assay procedure is not resolved by the present studies. By far, the most potent Ah receptor agonist identified in the reaction mixture is ICZ. Because of the higher yields of the weaker binding oligomers, ICZ appears of roughly equal importance to the other oligomers in the *in vivo* enzyme-inducing activity of the mixture. Comparisons of ICZ and TCDD indicate that both have high affinities for the Ah-receptor protein, have low toxicities for the Hepa 1c1c7 cells, and are potent inducers of CYP1A1 in cell culture. The difference we observe in inducing EC_{50} values for ICZ and TCDD from EROD assay (≈7000 fold) is larger than would be predicted

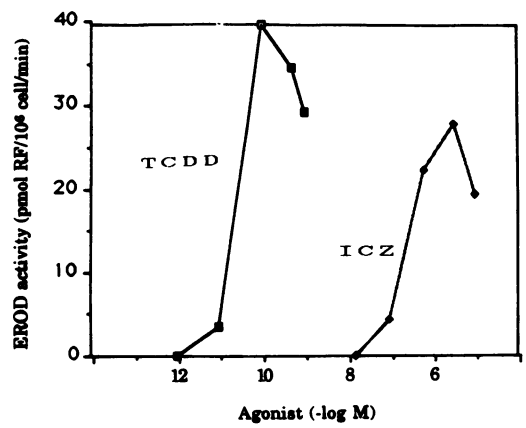

FIG. 7. Ethoxyresorufin *O*-deethylase activity (EROD) was determined in Hepa 1c1c7 cells as described. Computed mean EC_{50} values are 36 ± 12 pM and 260 ± 50 nM for TCDD and ICZ, respectively. (EC_{50} values are means ± SDs of three determinations for TCDD and five determinations for ICZ.)

from the observed differences in *in vitro* binding affinities (3.7 $\times 10^{-2}$ fold) and may arise from, among other causes, variations in cellular uptake, metabolic rate, or energy-dependent inducer efflux associated with multidrug resistance for the two compounds (34).

Because the Ah-receptor-binding and the CYP1A1-inducing characteristics of ICZ and TCDD are similar, their biological effects may be similar in other respects also. Indeed, TCDD and ICZ are both active in reducing lymphoid development in murine fetal thymus organ culture, although ICZ is less toxic than TCDD in this assay by a factor of 10^{-5} (35). Of special note in the context of the present investigation are the established activities of TCDD as an anti-initiator and as a promoter of carcinogenesis (36, 37). Similar cancer-related effects of ICZ or related oligomers may account for some of the cancer-modulating activities of I3C.

Because ICZ and TCDD may produce similar biological effects, it is of interest to compare a typical dietary dose of ICZ to the currently acceptable maximum dose of TCDD. Assuming that a 20% conversion of glucobrassicin to I3C occurs during maceration of plant material (2), a 100-g portion of brussels sprouts can provide between 10–50 μmol of I3C (38). A yield of 0.01% in the gastrointestinal tract, as indicated by the present studies, would provide a dose of 1–5 nmol (256–1280 ng) of ICZ. This dose is considerably in excess of the maximum acceptable daily human dose for TCDD established by the U.S. Environmental Protection Agency—i.e., 1.25 fmol (400 fg) for a 70-kg person. However, useful quantitative comparisons of the relative hazard, or benefit, of the two compounds as, for example, cancer modulators, cannot be made on the basis of available information. Affinity for the Ah-receptor protein *in vitro* and potency as an inducer of cytochrome P-450-dependent monooxygenases in cell culture may not be reliable indicators of results from long-term cancer tests in animals. Other factors to be considered include biological half-life, which may be as long as 10 yr for TCDD (39). The half-life of ICZ may be considerably shorter than this for several reasons, including the possibilities that ICZ may have less affinity for cellular storage sites than TCDD, ICZ may be metabolized to excretable products more rapidly than TCDD, and ICZ may be a better substrate for the multidrug resistance active-transport system than is TCDD (34). Although further studies are required to characterize the biological activity of ICZ, it appears unlikely that normal levels of ICZ from the diet are of significant hazard compared with the benefits of the micronutrients in *Brassica* vegetables (4).

We thank O. Hankinson, University of California, Los Angeles, for the gift of the Hepa 1c1c7 cells; I.-S. Kim, California Department of Public Health, Berkeley, CA, for HPLC-MS analyses; and J. Bergman, Royal Institute of Technology, Stockholm, Sweden, for an authentic sample of ICZ. C.B. is the recipient of a Junior Faculty Research Award (JFRA-303) from the American Cancer Society.

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